Effect of Osmolarity and Dehydration on Alginate Production by Fluorescent Pseudomonads

Abstract. Alginate is produced as an exopolysaccharide by many fluorescent pseudomonads. However, pseudomonads often have a nonmucoid phenotype in standard laboratory media. Growth in the presence of 0.3 M sodium chloride or 3–5% ethanol reportedly can lead to the generation of mucoid variants of nonmucoid strains of *Pseudomonas aeruginosa*. We wished to determine whether alginate production by other fluorescent pseudomonads is affected by sodium chloride and ethanol. Eight alginate-producing strains of saprophytic and phytopathogenic pseudomonads were grown as broth cultures containing 0–0.7 M sodium chloride or 0–5% ethanol for 24–30 h at 28° or 35°C. Culture supernatant fluids were subjected to ethanol precipitation, and the amount of alginate present was estimated by measuring the uronic acid content. The presence of sodium chloride and ethanol caused significant stimulation of alginate production by all strains tested except *P. viridiflava* ATCC13223 and *P. fluorescens* W4F1080. The optimal concentration of sodium chloride ranged from 0.2 to 0.5 M; that for ethanol ranged from 1 to 3%. Moreover, inclusion of the nonmetabolizable, nonionic solute sorbitol showed a similar stimulation of alginate production. The stimulation of alginate production by high medium osmolarity and dehydration appears to be a trait shared by fluorescent pseudomonads.

Alginate is a 1,4-linked linear co-polymer of beta-D-mannuronic and alpha-L-guluronic acids [16]. Alginate isolated from brown algae is used for a variety of commercial purposes. It is also produced as a bacterial exopolysaccharide by mucoid strains of *Pseudomonas aeruginosa* isolated from the lungs of cystic fibrosis patients [8] and by strains of *Azotobacter vinelandii* isolated from a variety of natural habitats [19]. Variants of *P. fluorescens*, *P. putida*, and *P. mendocina* that produce alginate can be generated by a variety of means [13, 15]. More recently, several naturally occurring strains of saprophytic as well as plant pathogenic fluorescent pseudomonads were shown to be capable of alginate production [10, 11, 14].

Alginate produced by mucoid strains of *P. aeruginosa* in the lungs of cystic fibrosis patients is thought to function as a virulence factor by interfering with phagocytosis [22], by providing a barrier against penetration of antibiotics [24], and by exasperating bronchial obstruction [7]. Alginic acid produced by saprophytic and plant pathogenic fluorescent pseudomonads may play a role in adhesion to plant surfaces, concentration of cations, protection against adverse environmental conditions, and as a virulence factor towards plants [3, 9].

Regulation of alginate synthesis by mucoid strains of *P. aeruginosa* is the subject of continuing intense investigation because of the tragic fatal consequences of chronic lung infections by this bacterium in cystic fibrosis patients. This regulation is highly complex, with a number of positive and negative regulatory genes known [5, 20]. Environmental signals reported to induce mucoidy in this bacterium include increased osmolarity as well as dehydration [1, 6]. The environmental signals that induce alginate production by saprophytic and plant pathogenic bacteria are as yet completely unknown. This study represents the first attempt at determining such factors for this large group of bacteria. Here we report on the effect of increased medium osmolarity and of dehydration on alginate production *in vitro* by

Table 1. Strains of fluorescent pseudomonads used

Bacterium	Strain	Phytopathogenic	Reference/ source
P. syringea pv. *glycinea*	A-29-2m	+	23
	NCPPB2159	+	23
P. viridiflava	671m	+	F. Lukezic
	ATCC13223[a]	+	ATCC
P. fluorescens	W4F 1080	−	11
	R8Z-80	−	11
	R4a-80	−	11
	W4F 1607	−	11

[a] ATCC, American Type Culture Collection, Rockville, Maryland, USA.

selected representatives of the fluorescent pseudomonads.

Materials and Methods

Strains. The fluorescent pseudomonad strains used in this study are listed in Table 1. All strains are capable of producing alginate as an exopolysaccharide under certain culture conditions [11, 23, Fett unpublished]. Strains designated by "m" are single-colony clones from the original cultures that exhibit a mucoid phenotype when grown on *Pseudomonas* agar F (Difco Laboratories). Bacteria were maintained on this medium at 4°C.

Growth media and culture conditions. Bacteria were cultured in a modified King's medium B broth [18] with Bacto tryptone (Difco) substituted for proteose peptone #3 and addition of NaCl (0–0.7 M) or ethanol (0–5%). Two ml of starter culture grown overnight was used to inoculate 25 ml of broth contained in 250-ml Erlenmeyer flasks. Flasks were shaken (200 rpm) for 24–30 h at 28°C (*P. syringae* pv. *glycinea* and *P. viridiflava*) or 35°C (*P. fluorescens*), at which time cultures in modified King's medium B alone had reached stationary phase based on dry cell weight measurements.

Cells were harvested by centrifugation (7800 *g*, 15 min) in preweighed centrifuge tubes and the supernatant fluids collected. The cells were washed once with 5 ml of sterile water, and the final cell pellets were dried to a constant weight at 80°C. The culture supernatant fluids plus the cell washes were combined and subjected to precipitation with ice-cold ethanol (3 vol) after addition of KCl to a final concentration of 1%. After 30 min of stirring, the resultant precipitate was collected by centrifugation (17,000 *g*, 15 min), suspended in 5 ml of distilled water, and vigorously mixed for 2 min. Any insoluble material was then removed from the samples by centrifugation (17,000 *g*, 15 min).

Determination of alginate content. Alginate content of the samples was determined by use of a colorimetric assay for uronic acids [2] with a commercial alginate sample (Sigma) used to generate standard curves.

To confirm the presence of alginate, a sample obtained from *P. syringae* pv. *glycinea* A-29-2m was dialyzed twice against 10^3 volumes of distilled water, and the retentate was lyophilized. The dried sample was then reduced, derivatized to give the aldononitrile acetate form and analyzed by gas-liquid chromatography as previously described [23].

Results and Discussion

P. syringae pv. *glycinea* A-29-2m, *P. viridiflava* 671m and ATCC 13223, as well as *P. fluorescens* R8z-80 were relatively insensitive to inhibition by addition of NaCl with 35% or less reduction in cell dry weight at 0.5 M NaCl. The most sensitive strains were *P. syringae* pv. *glycinea* NCPPB 2159, and *P. fluorescens* R4a-80, which showed a 75% or greater reduction in cell dry weight when NaCl was present at 0.5 M.

All strains except for *P. viridiflava* ATCC 13223 and *P. fluorescens* W4F1607 produced detectable amounts of alginate in the broth medium without any additions (Table 2). Gas-liquid chromatography of the aldononitrile derivatives of reduced and unreduced samples from *P. syringae* pv. *glycinea* A-29-2m confirmed the presence of alginate. The alginate from strain A-29-2m was determined to be a polymannuronan with no detectable guluronic acid present. Addition of NaCl to the medium caused increased production of alginate by the six strains that produced alginate in broth medium alone, but did not induce production of alginate by *P. viridiflava* ATCC 13223 and *P. fluorescens* W4F1607. Optimal concentrations of NaCl ranged from 0.2 to 0.5 M. The greatest increase (22-fold) in alginate production on a cell dry weight basis occurred for *P. fluorescens* R4a-80 with NaCl at 0.4 M. On a mg of alginate/L basis, the greatest increase (8-fold) was seen for *P. syringae* pv. *glycinea* A-29-2m with NaCl at 0.5 M (Table 2).

To distinguish between an ionic versus an osmotic stimulator effect due to addition of NaCl to the broth medium, the nonionic, nonmetabolizable solute sorbitol was also examined for its effect on alginate production by *P. syringae* pv. *glycinea* A-29-2m and NCPPB 2159. For these experiments, the effect of sorbitol at 0.8 M was compared with the effect of NaCl at 0.4 M (osmotically equivalent concentrations). Cultures were incubated for 48 h. Addition of sorbitol had no inhibitory effect on growth for either strain, but did lead to increased production of alginate to an extent similar to that which occurred with addition of NaCl (Table 3).

The variation in inhibition of bacterial growth by addition of ethanol to the medium was not so great as seen for NaCl. At 3%, ethanol caused between a 30% and 60% reduction in cell dry weights. The greatest increase (16-fold) in alginate production oc-

Table 2. Effect of sodium chloride on alginate production

Bacterium	μg of alginic acid/mg dry cell wt. (mg of alginic acid/liter)							
	Concentration of sodium chloride in medium							
	0.0 M	0.1 M	0.2 M	0.3 M	0.4 M	0.5 M	0.6 M	0.7 M
P. syringae pv. *glycinea*								
A-29-2m	12.7 ± 5.5	16.4 ± 3.6	34.5 ± 15.9	51.92 ± 25.5	84.9 ± 42.3	116.7 ± 32.8	100.1 ± 33.8	79.0 ± 29.0
	(63 ± 31)	(86 ± 30)	(185 ± 95)	(257 ± 127)	(394 ± 186)	(478 ± 102)	(284 ± 48)	(88 ± 46)
NCPPB 2159	48.6 ± 5.1	58.8 ± 9.3	138.7 ± 24.3	284.4 ± 148.7	358.6 ± 241.4	181.6 ± 85.6	186.4 ± 90.7	125.0 ± 53.6
	(266 ± 26)	(254 ± 46)	(436 ± 216)	(852 ± 548)	(784 ± 512)	(264 ± 152)	(238 ± 150)	(130 ± 7)
P. viridiflava								
671m	2.8 ± 0.7	6.3 ± 1.4	8.1 ± 0.1	12.0 ± 4.2	8.2 ± 2.8	6.7 ± 2.7	NT	NT
	(16 ± 4)	(36 ± 9)	(61 ± 19)	(63 ± 23)	(38 ± 14)	(31 ± 13)		
ATCC 13223	ND	ND	ND	ND	ND	ND	ND	ND
P. fluorescens								
W4F 1080	1.0 ± 0.3	1.2 ± 0.1	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.4	1.3 ± 0.3	NT	NT
	(6 ± 2)	(7 ± 1)	(8 ± 1)	(8 ± 1)	(7 ± 2)	(5 ± 1)		
R8Z-80	0.8 ± 0.4	1.2 ± 0.6	3.2 ± 2.0	2.6 ± 0.5	2.1 ± 0.1	1.9 ± 0.1	NT	NT
	(6 ± 2)	(9 ± 4)	(24 ± 11)	(21 ± 0)	(15 ± 3)	(13 ± 3)		
R4a-80	3.7 ± 1.4	12.4 ± 0.3	22.1 ± 4.2	58.2 ± 25.5	81.0 ± 12.5	71.5 ± 0.1	NT	NT
	(22 ± 8)	(73 ± 3)	(122 ± 34)	(139 ± 5)	(121 ± 5)	(105 ± 21)		
W4F 1607	ND	ND	ND	ND	ND	ND	NT	NT

Each result is an average value from two experiments ± standard deviation.
ND = none detected. NT = not tested.

Table 3. Effect of ionic (NaCl) and nonionic (sorbitol) solutes on alginate production by *Pseudomonas syringae* pv. *glycinea*

Solute	Strains	
	A-29-2m	NCPPB2159
Control		
μg/mg dry cell wt	32 ± 0.4	98 ± 44.0
mg/L	194 ± 2.0	602 ± 206.0
Sorbitol (0.8 M)		
μg/mg dry cell wt	171 ± 1.0	287 ± 8.0
mg/L	1116 ± 124.0	1616 ± 0.0
NaCl (0.4 M)		
μg/mg dry cell wt	179 ± 38.5	269 ± 50.0
mg/L	1256 ± 136.0	1176 ± 360.0

Values are averages from two experiments ± standard deviation.

curred for *P. viridiflava* 671m with ethanol at 3% (Table 4). This strain also showed the greatest increase (12-fold) on an mg of alginate/L basis when ethanol was added to 2% (Table 4).

In this report we directly examined the influence of sodium chloride (ionic or osmotic effect), sorbitol (osmotic effect), and ethanol (dehydrating agent) on alginate biosynthesis by both plant pathogenic and saprophytic fluorescent pseudomonads. For the majority of the strains, highest alginate production was attained after addition of NaCl or ethanol in amounts which led to reduced bacterial growth based on dry cell weight measurements. However, addition of sorbitol to the culture medium also increased alginate production without a concomitant reduction in growth.

There have been several reports on the effect of increased osmolarity on alginate production by *P. aeruginosa*. Growth of nonmucoid *P. aeruginosa* PAO1 in continuous culture in the presence of 0.3 M NaCl resulted in the generation of mucoid variants [25]. For the stable mucoid, mutant strain *P. aeruginosa* 8821, more than a fourfold increase in expression of the *algD* gene, a gene that encodes for the key enzyme (GDP-mannose dehydrogenase) involved in alginate biosynthesis, occurred when NaCl was added to broth cultures [1]. The optimum concentration of NaCl was reported to be 0.35 M. However, even though transcription of *algD* in the nonmucoid strain PAO1 was increased threefold under conditions of high osmolarity, no increase in alginate production resulted, indicating that additional environmental factors are involved in the conversion to a mucoid phenotype [1]. Clinical strains of *P. aeruginosa* are reported to be very diverse in their response to medium osmolarity, with a majority showing no response [5].

There have been no reports on the effect of osmolarity on alginate production by pseudomonads other than *P. aeruginosa*. Our results indicate that

Table 4. Effect of ethanol on alginate production

Bacterium	μg of alginic acid/mg dry cell wt. (mg of alginic acid/liter)					
	Concentration of ethanol in medium					
	0%	1%	2%	3%	4%	5%
P. syringae pv. *glycinea*						
A-29-2m	19.6 ± 1.8	53.4 ± 5.6	81.4 ± 8.1	116.9 ± 7.5	83.0 ± 5.9	52.7 ± 7.3
	(118 ± 18)	(264 ± 48)	(349 ± 41)	(434 ± 14)	(154 ± 6)	(44 ± 4)
NCPPB 2159	18.7 ± 2.2	35.9 ± 2.6	63.1 ± 6.0	79.4 ± 2.5	37.1 ± 2.3	43.4 ± 6.6
	(100 ± 16)	(150 ± 30)	(210 ± 50)	(170 ± 10)	(51 ± 1)	(34 ± 6)
P. viridiflava						
671m	4.8 ± 1.1	44.4 ± 24.1	69.4 ± 4.7	76.1 ± 2.8	32.0 ± 6.5	18.2 ± 1.5
	(29 ± 7)	(231 ± 117)	(354 ± 10)	(338 ± 22)	(69 ± 11)	(18 ± 4)
ATCC 13223	ND	ND	ND	ND	ND	ND
P. fluorescens						
W4F 1080	3.9 ± 2.3	11.2 ± 9.1	8.1 ± 5.1	4.8 ± 0.7	2.9 ± 0.8	1.1 ± 0.0
	(34 ± 23)	(80 ± 70)	(37 ± 23)	(19 ± 3)	(7 ± 1)	(4 ± 0)
R8Z-80	2.9 ± 1.4	3.2 ± 1.9	4.5 ± 2.2	3.9 ± 1.3	2.6 ± 0.7	1.0 ± 0.0
	(22 ± 12)	(23 ± 14)	(30 ± 16)	(22 ± 8)	(11 ± 3)	(3 ± 0)
R4a-80	8.9 ± 0.9	24.5 ± 4.2	39.6 ± 10.4	51.6 ± 16.9	48.3 ± 13.6	41.7 ± 13.3
	(53 ± 10)	(89 ± 19)	(42 ± 7)	(38 ± 13)	(38 ± 13)	(34 ± 19)
W4F 1607	ND	ND	ND	ND	ND	ND

Each result is an average from two experiments ± standard deviation.
ND = none detected.

osmolarity can increase the amount of alginic acid produced by plant pathogenic as well as saprophytic fluorescent pseudomonads under the culture conditions utilized, but that the stimulatory effect is strain specific. Optimal concentrations of NaCl in our studies (0.2–0.5 M) were similar to those reported for *P. aeruginosa*, as discussed above. The stimulation of alginate synthesis by addition of NaCl to the medium appears to be owing to the resultant increase in osmolarity rather than an ionic effect based on our results with the nonionic solute sorbitol. In contrast, Terry et al. [25] concluded that the appearance of mucoid variants of nonmucoid *P. aeruginosa* PAO1 during continuous culture in the presence of NaCl was most likely an effect of increased ionic strength rather than increased osmolarity, on the basis of the inactivity of 0.4 M sucrose. However, Berry et al. [1] found similar levels of transcriptional activation of *algD* with iso-osmotic concentrations of sucrose and NaCl or KCl.

Based on our studies, dehydration also appears to stimulate alginate production by some, but not all, plant pathogenic and saprophytic fluorescent pseudomonads. DeVault et al. [6] found that addition of 1% ethanol to broth media caused a twofold increase in alginate production by mucoid *P. aeruginosa* strain 8821, but did not cause alginate to be produced by the nonmucoid strains PAO1 and 8822, a spontaneous nonmucoid revertant of strain 8821. However, addition of 3–5% ethanol to a solid medium did lead to the appearance of mucoid variants of the two nonmucoid strains.

The molecular basis for the stimulation of alginate production by *P. aeruginosa* because of increased osmolarity or dehydration may involve transcriptional activation of at least two genes, *algD* and *algR1* [20], although the involvement of *algR1* in either case is not entirely clear [5, 21]. The gene *algR1* appears to be the receiver component of a two-component signal transduction regulatory system, and activation of *algD* is dependent on the *algR1* gene product [5, 20]. Changes in osmolarity were reported to affect transcription of *algR1* as well as *algD*, in *muc* loci mutants of *P. aeruginosa* [4]. Transcription was either stimulated or inhibited by the presence of 0.3 M NaCl dependent on the particular *muc* gene mutation present. Kimbara and Chakrabarty [17] cloned a *algR1-xylE* promotor construct into *E. coli* and showed transcription was activated by NaCl. The sensory component of the putative two-component sensory transduction system in *P. aeruginosa* has yet to be identified. The product of the *algR1* gene may be required for activation of *algD* in response to ethanol [6],

but alternatively ethanol may be inducing a heat-shock response [5].

Based on Southern blot analysis, gene sequences with homology to *algR1* were shown to occur in alginate-producing fluorescent pseudomonads other than *P. aeruginosa* [12]. Thus, for the bacteria used in this study, a similar two-component sensory system may be functioning in the osmotic stimulation of alginate production. Similar two-component regulatory systems have been reported for plant pathogenic bacteria; e.g., *virG* and *virA* in *Agrobacterium tumefaciens* [26]. We reported that plant pathogenic fluorescent pseudomonads that were nonmucoid on standard agar medium were induced to produce alginate in leaves of their host plants [9]. Whether this induction was due to high osmolarity or dehydration within the leaf intercellular spaces where the bacteria reside is not known.

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